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Purification and Characterization of a New Indole Oxygenase from the Leaves of *Tecoma stans* L.

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ABSTRACT

A new indole oxygenase from the leaves of *Tecoma stans* was isolated and purified to homogenity. The purified enzyme system catalyzes the conversion of indole to anthranilic acid. It is optimally active at pH 5.2 and 30°C. Two moles of oxygen are consumed and one mole of anthranilic acid is formed for every mole of indole oxidized. Dialysis resulted in complete loss of the activity. The inactive enzyme could be reactivated by the addition of concentrated dialysate. The enzyme is not inhibited by copper-specific chelators, non-heme iron chelators or atebrin. It is not a cuproflavoprotein, unlike the other indole oxygenases and oxidases.

The physiological importance of the indole ring has generated considerable interest in the biological transformation of indole derivatives. Abnormal metabolism of indole has been found in phenylketonuria, Hartnup disease, and pellagra (15) and also in certain mental disorders (14). However, reports on the metabolism of indole are few and far between.

Indole has been reported to be metabolized by two types of bacterial isolates, one from tap water (12) and the other from soil (5). Metabolism of this compound was achieved by its conversion to anthranilic acid via indoxyl, isatin, and *N*-formylanthranilic acid in the tap water bacterium, while the soil organism carried out this oxidation with the intermediate formation of dihydroxyindole.

An enzyme system which converts indole to anthranil (3,4-benzoisoxazol) via *N*-formylaminobenzaldehyde has been reported in crude extracts from the leaves of *Tecoma stans* (9). An enzyme which oxidizes indole to anthranilic acid, apparently without any intermediate formation, was purified from the leaves of *Jasminum grandiflorum* (4). Chauhan *et al.* (2) have detected an indole-oxidizing enzyme system in the leaves of *Zea mays*. This enzyme oxidizes indole to both anthranil and anthranilic acid. All these enzymes from plant sources are cuproflavoproteins. This paper describes a novel indole oxygenase from *T. stans*, which catalyzes the conversion of indole to anthranilic acid.

MATERIALS AND METHODS

Chemicals. Indole, anthranilic acid, indoleacetic acid, tryptophan, Tris, pyridoxal 5-P, GSH, 2-methylindole, 5-methylindole, 5-hydroxyindole, 5-bromoindole, 7-methylindole, *p*-chloromercuribenzoate, *N*-ethylamaleimide, idoacetate, DTT, *o*-phenanthroline, 8-hydroxyquinoline, salicyladoxime, diethyldithiocarbamate, bathophenanthroline sulfate, FAD, FMN, NADH, NADPH, THFA,[2] biopterin, and atebrin were purchased from Sigma. CM-cellulose and Sephadex G-100 were procured from Pharmacia Fine Chemicals, Uppsala, Sweden. *o*-Aminobenzaldehyde was prepared from *o*-nitrobenzaldehyde by selective reduction according to the method of Smith and Opie (13). All other chemicals used were of analytical grade available commercially.

Assay of Indole Oxygenase. A reaction mixture (2.0 ml) consisting of 100 nmol indole, 1.4 ml 0.1 M citric acid-0.2 M sodium phosphate buffer, pH 5.2, and 100 μg enzyme was incubated at 30°C. After 15 min, the reaction was stopped by shaking with 2.5 ml toluene, which extracted the unreacted indole. The layers were separated by centrifugation at 3000*g* for 5 min and 1 ml of the toluene layer was used for the estimation of indole using Ehrlich reagent (16).

One unit of enzyme activity is defined as that amount of the enzyme which causes the disappearance of 1 nmol indole in 1 min at 30°C. Specific activity is expressed as units/mg protein.

Determination of the Approximate Molecular Weight of the Enzyme. The approximate mol wt of indole oxygenase was determined by gel filtration on a Sephadex G-100 column according to the procedure of Andrews (1) and also by SDS gel electrophoresis (19).

The following proteins of known mol wt served as markers in both the procedures: ovalbumin (46,000), BSA (68,000), Cyt *c* (12,270), chymotrypsinogen (23,200), and hexokinase (102,000).

Polyacrylamide-Gel Disc Electrophoresis. Analytical gel electrophoresis was carried out by the procedure described by Davis (3) on 7.5% (w/v) gels at 6 mamp/gel and continued until the dye band reached the lower end of the gel. For analysis of indole oxygenase, 0.1 M Tris/glycine buffer (pH 8.3) was used. The proteins on the gels were stained with Coomassie Brilliant Blue R-250 and destained with methanol/acetic acid/water (50:7:43, v/v).

Protein Estimation. Proteins were precipitated with ethanol and centrifuged at 3000*g* for 5 min. The precipitate was redissolved in buffer and the protein content of various preparations was estimated by the method of Lowry *et al.* (8) using BSA as the standard.

Estimation of Anthranilic Acid. The unreacted indole was extracted from the reaction mixture (2.0 ml) with 2.5 ml toluene, and anthranilic acid was estimated in a 0.5-ml aliquot of the aqueous phase according to the method of Venkataraman *et al.* (18) using Ehrlich reagent.

Immunochemical Techniques. Antibodies to the purified indole oxygenase were raised in albino rabbits. The protein dissolved in 10 mM sodium phosphate buffer (pH 7.2)/0.15 M NaCl (1 mg/ml) and emulsified with an equal volume of Freund's complete adju-

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[2] Abbreviation: THFA, tetrahydrofolic acid.

vant (Difco Laboratories) was injected subcutaneously at multiple points at weekly intervals. After 4 weeks, a booster dose of 0.5 mg protein in 10 mM sodium phosphate/0.15 M NaCl was administered. After 10 more days, the rabbits were bled through the ear vein, and serum was prepared. Double immunodiffusion analysis on agar was performed as described by Ouchterlony (11).

Isolation of the Reaction Products. A reaction mixture consisting of 4 ml enzyme (2.4 mg protein), 3 μmol indole, and 12 ml 10 mM citric acid/0.2 M sodium phosphate buffer (pH 5.2) was incubated for 30 min at 30°C. At the end of the incubation period, unreacted indole was extracted with toluene, the pH of the reaction mixture was adjusted to 2 with 2 N HCl, and the contents were extracted twice with peroxide-free diethyl ether. The ether layers were pooled, dried over anhydrous sodium sulfate, concentrated to a small volume, and subjected to paper chromatography on Whatman No. 3 filter paper using ethanol/ammonia/water (18:1:1, v/v) as the solvent system. The product of the reaction mixture was located on the chromatograms by its fluorescence and also by its color reaction with Ehrlich reagent. It was eluted with ethanol and identified as anthranilic acid by comparing its R_F values in different solvent systems, color reaction with Ehrlich reagent and UV, fluorescence and IR spectra with those of an authentic sample.

Subcellular Fractionation. Various subcellular fractions of *Tecoma* leaves were prepared according to the method of Nair and Vaidyanathan (10).

Instrumentation. UV absorption spectra were recorded on a Beckman model 26 UV spectrophotometer and fluorescence spectra were recorded on a Perkin-Elmer spectrofluorimeter model 203. IR spectra were recorded on a Perkin-Elmer infrared spectrophotometer model 700, using the nujol mull technique. O_2 uptake studies were carried out with a Gilson Oxygraph.

RESULTS

Purification of Indole Oxygenase from Tecoma Leaves. All operations were carried out at 0 to 5°C, unless otherwise stated. In the first step, fresh, mature leaves (50 g) of *Tecoma stans* L. were taken, and the midribs from the leaves were detached and washed thoroughly with cold glass-distilled H_2O. The leaves were then ground to a paste in a chilled porcelain mortar with acid-washed glass powder (10 g) and the slurry was extracted with 90 ml of 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2. The slurry was strained through cheesecloth and the greenish extract obtained was centrifuged at 12,000*g* for 15 min in a refrigerated centrifuge. To the clear supernatant, designated as the crude enzyme, 9 ml 1 M manganese sulfate solution were added drop by drop with gentle stirring. After 10 min, the extract was centrifuged at 12,000*g* for 10 min to get a clear supernatant fraction. Subsequently, this supernatant solution was taken to 30% saturation by the addition of solid ammonium sulfate with constant stirring and set aside for 15 min. The precipitate obtained on centrifugation at 12,000*g* for 10 min was discarded. To the supernatant solution, solid ammonium sulfate was added to raise the saturation level to 50% and it was set aside for 30 min. The precipitate obtained on centrifugation at 12,000*g* for 10 min was dissolved in a minimum amount of 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2, and was designated as the 30 to 50% ammonium sulfate fraction.

In a fourth step, the 30 to 50% ammonium sulfate fraction was passed through a Sephadex G-25 column (1.4 × 32 cm) previously equilibrated with 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2, to remove ammonium sulfate from the preparation. Active fractions (8 ml) were pooled and passed through a CM-cellulose column (1.5 × 25 cm) previously equilibrated with 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2. Active fractions (20 ml) were pooled and used for the next step. Finally, 10.0 ml tricalcium phosphate gel (15.2 mg/ml) solution previously equilibrated with 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2, was centrifuged at 1000*g* for 3 min and the enzyme preparation from the fourth step (20 ml) was added to it. The gel was uniformly suspended in the enzyme solution and allowed to equilibrate for 15 min. The gel solution was then centrifuged at 1000*g* for 3 min and the supernatant was discarded. The brown gel was washed twice with 10 mM citric acid-20 mM sodium phosphate buffer, pH 5.2, and the washings were rejected. The

Table I. *Purification of Indole Oxygenase from Tecoma stans L.*

Preparation	Total Activity	Specific Activity	Purification	Recovery
	units	*units/mg protein*	*-fold*	*%*
Crude	109	0.13	1.0	100.0
Manganese sulfate treatment	106	0.25	1.9	97.0
30%–50% ammonium sulfate (desalted)	72.2	2.73	21.0	66.3
CM-cellulose	55.2	14.70	113.0	32.3
Tricalcium phosphate gel	18.8	15.70	121.0	17.3

Table II. *Identification of the Product of the Indole Oxygenase Reaction*

Property	Enzymic Product	Authentic Product
R_F value in		
Isopropanol/ammonia/water (20:1:2)	0.35	0.33
Butanol/acetic acid/water (4:1:1)	0.88	0.88
Benzene/acetic acid/water (10:7:3)[a]	0.95	0.95
Ethanol/ammonia/water (18:1:1)	0.52	0.53
Formic acid/water (2:98)	0.86	0.86
λ_{max} in ethanol (nm)	221	221
	247	247
	335	335
Color reaction with Ehrlich's reagent	Yellow	Yellow
λ_{max} (nm)	450–470 (broad)	450–470 (broad)
Ultraviolet fluorescence (nm; solvent: ethanol)		
Excitation maxima	312	312
	365	365
Emission maximum	405	405

[a] Mixed in given proportion (v/v) and only organic phase was used.

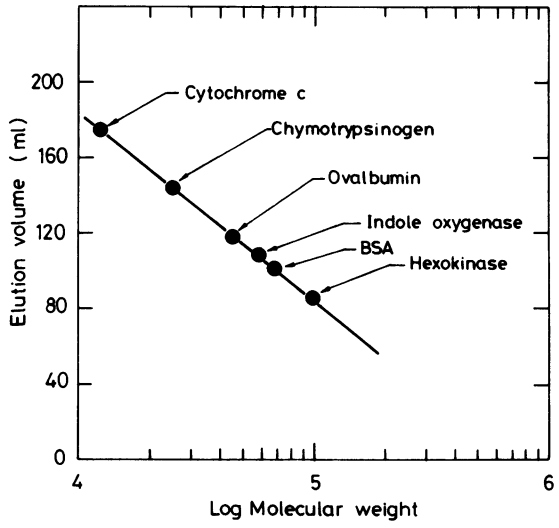

FIG. 1. Molecular weight determination by gel filtration on Sephadex G-100.

enzyme was finally eluted from the gel with 0.1 M citric acid-0.2 M sodium phosphate buffer, pH 5.2, and was used as the enzyme source throughout the course of the investigation.

By following the purification procedure outlined above, a 121-fold purified enzyme was obtained with 17% recovery. A summary of the purification procedure is given in Table I.

Analysis of the purified enzyme by polyacrylamide gel electrophoresis at pH 8.3 revealed a single protein band. On Ouchterlony plates, with antiserum in the central well and the enzyme preparation in the surrounding wells, only one precipitin line was observed, confirming the homogeneous nature of the enzyme.

Characterization of the Enzymic Product. The enzymic product was identified as anthranilic acid by comparing its chromatographic mobilities and spectral properties with those of an authentic sample. The R_F values in different solvent systems and spectral properties of both enzymic products and the authentic anthranilic acid are given in Table II. The IR spectra of the enzymic product and authentic anthranilic acid were superimposable. Formic acid, the other possible product of the indole oxygenase reaction, could not be detected. No anthranil was detected in the reaction mixture.

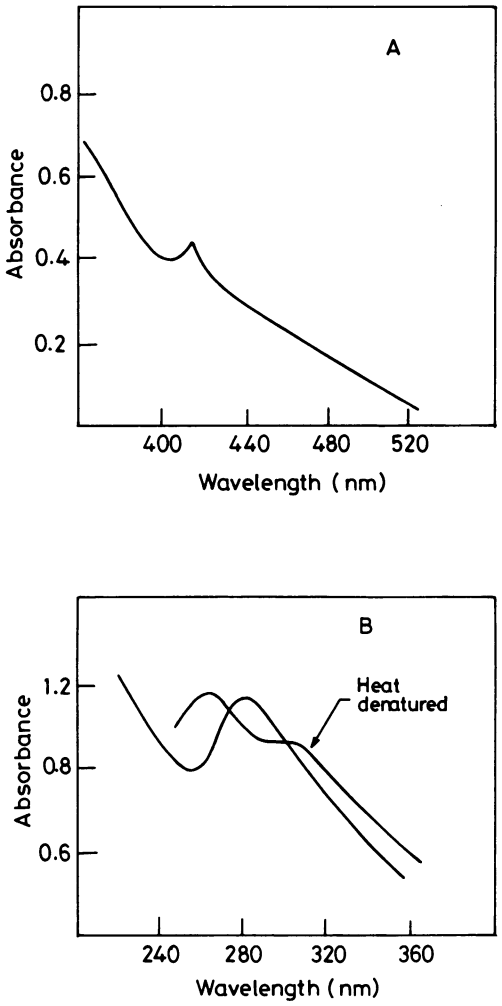

FIG. 2. Absorption spectra of indole oxygenase in the visible region (A) and in the UV region (B). Concentration of the enzyme was 2 mg/ml (A) and 1 mg/ml (B) in citric acid-sodium phosphate buffer, pH 5.2. Heat treatment was carried out at 60°C for 3 min with 1 mg enzyme.

Table III. *Requirement for Molecular Oxygen*

Anaerobic experiments were carried out in Thunberg tubes after alternately flushing with N_2 and evacuating. Standard assay mixture was scaled up two times and used.

Atmosphere	Indole Disappearance
	nmol
Aerobic	40
Anaerobic	0
Air let in after 15 min	36

Table IV. *Stoichiometry of the Reaction Catalyzed by Indole Oxygenase from Tecoma stans*

Time	Indole Disappeared	O_2 Consumed	Anthranilic Acid Formed
min		*nmol*	
10	16	31	16
12	20	39	19
15	24	48	23

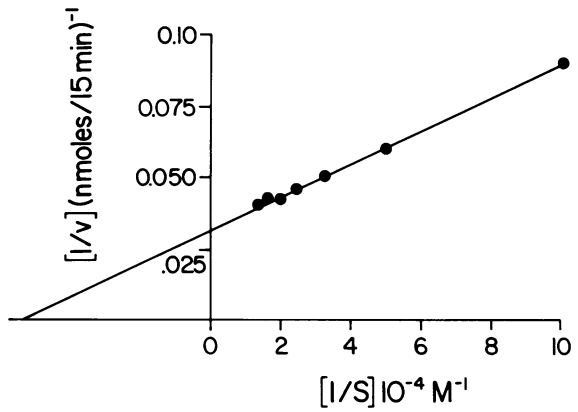

FIG. 3. Lineweaver-Burk plot. The concentration of the substrate was varied in the standard assay mixture.

Characteristics of the Enzyme. The purified enzyme can be stored at −20°C only for approximately 18 h without appreciable loss of activity. Its activity is completely lost after 48 h at −20°C. The mol wt of the enzyme as determined by gel filtration on Sephadex G-100 (Fig. 1) and on SDS gel electrophoresis is 58,000 and it consists of a single polypeptide chain.

The absorption spectra of the enzyme in the visible region and UV region are shown in Figure 2. In the UV region, the enzyme exhibits a typical protein spectrum. The visible spectrum of the enzyme shows a peak at 408 nm, which may be due to bound cofactors. Addition of the substrate, indole, under aerobic conditions to the enzyme caused no change in the spectrum of the enzyme in the visible region. However, on incubation of the enzyme at 60°C for 3 min, there is a shift in the UV spectrum of the enzyme, which may be due to the exposure of tyrosine and tryptophan residues.

The enzyme is optimally active at pH 5.2 in 0.1 M citric acid-0.2 M sodium phosphate buffer and at 30°C. The reaction catalyzed by indole oxygenase was found to be linear up to 15 min of the incubation period and 120 μg of the protein concentration. The enzyme shows an absolute requirement for molecular oxygen as evident from the results shown in Table III. The enzyme was completely inhibited by 1 mM dithionite, suggesting the oxidative nature of indole disappearance. The stoichiometry of the reaction catalyzed by indole oxygenase is presented in Table IV. Two moles of oxygen are consumed and 1 mol of anthranilic acid is formed for every mol of indole that disappears.

A study of the effect of different concentrations of indole on the rate of its disappearance gave hyperbolic curves. The K_m value for the substrate was determined by a Lineweaver-Burk plot to be 18.9 μm (Fig. 3). The substrate specificity of indole oxygenase was investigated and the results are given in Table V. A perusal of the

results shows that the enzyme has a comparatively wide specificity.

The response of the *Tecoma* indole oxygenase to various metal ions was studied. Activity was not affected by supplementing the enzyme with any of the following salts: (1.0 and 0.5 mM), $FeCl_3$, $MnSo_4$, $CoCl_2$, $ZnSo_4$, and $NiSo_4$. Of the different metal ions tested, only $FeSo_4$ and $CuSo_4$ caused 50% and 25% inhibition at 1 mM, respectively. The effects of various thiol compounds such as GSH, DTT, and L-cysteine and thiol reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetate, and mercuric chloride were studied. At 1 mM, none of them inhibits enzyme activity. To learn whether indole oxygenase behaves similarly to tryptophan peroxidase (7), the effect of beef liver catalase (1000 units) on the enzyme activity was studied. It had no effect on indole oxygenase.

Characteristics of the Cofactors. With a view to understanding the nature of cofactors, the effect of various cofactors such as NADPH, NADH, FMN, FAD, THFA, biopterin, pyridoxal phosphate, and ascorbic acid on the enzyme activity was studied and it was found that none of them caused any activation. This suggests that the enzyme by itself has bound cofactors and does not require any exogenous cofactors. All the other indole-oxidizing enzymes from plant sources, including the one from the same source, are activated by FAD and are completely inhibited by 1 mM atebrin. But indole oxygenase from *T. stans* was not inhibited to any extent by atebrin at 1 mM, suggesting that it may not be a flavoprotein. The effect of various metal chelators on enzyme activity was investigated and it was found that at 0.5 mM concentration only cyanide, azide, and carbon monoxide caused 64%, 54%, and 58% inhibition, respectively. The enzyme activity was not inhibited by copper-specific chelators such as diethyldithiocarbamate, salicylaldoxime bathocuproine and neocuproine, and non-heme iron (Fe^{2+})-specific chelators such as *o*-phenanthroline, bathophenanthroline sulfate, and α,α'-dipyridyl at 0.5 mM.

The enzyme loses its activity completely on dialysis for 8 h. The dialyzed enzyme was not reactivated by any of the following metal ions and cofactors at 0.5 mM: Cu^{2+}, Fe^{2+}, Mn^{2+}, Mn^{2+}, FAD, FMN, NADPH, NADH, THFA, biopterin, and ascorbic acid. The dialyzed enzyme could not be reconstituted by the addition of any combination of metal ions and cofactors. It could only be reactivated by the addition of concentrated dialysate, suggesting the loss of endogenous cofactors during dialysis.

Table V. *Substrate Specificity of Indole Oxygenase*

Each of the compounds was used as substrate and the enzyme was assayed under standard conditions. Enzyme activity with indole as substrate was taken as 100% and other compounds were compared.

Compound Added	Relative Activity
Indole	100
2-Methylindole	0
3-Methylindole	0
Tryptophan	0
5-Bromoindole	15
5-Hydroxyindole	20
5-Methylindole	26
7-Methylindole	0
Isatin	0

Table VI. *Subcellular Distribution of the Indole Oxygenase in Tecoma Leaves*

The assay conditions employed were standard except that the purified enzyme was replaced by 0.5 ml of the fractions in each case. The pellet was resuspended in 10 mM citric acid-20 mM Na phosphate buffer containing 0.35 M NaCl.

Fraction	Indole Disappeared
	nmol/15 min
Chloroplast fraction (1,000*g* pellet)	3
Mitochondrial fraction (20,000*g* pellet)	0
Microsomal fraction (105,000*g* pellet)	0
Soluble fraction (105,000*g* supernatant)	23

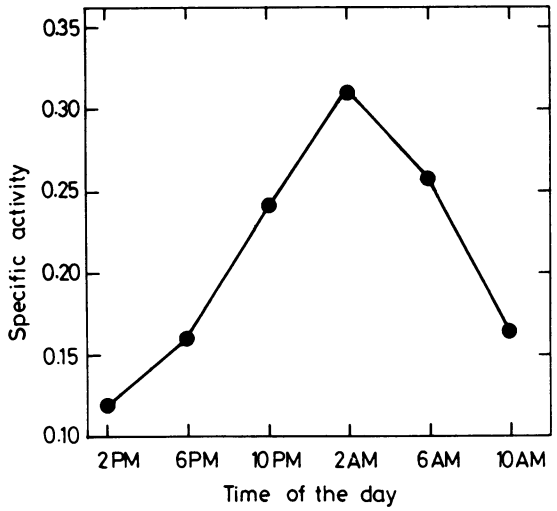

FIG. 4. Diurnal variation of indole oxygenase in the leaves of *T. stans*. The leaves were harvested at different times in a day and the enzyme was purified up to step 2 and assayed under standard conditions.

Physiological Characteristics of Indole Oxygenase. Table VI shows the distribution of indole oxygenase in different subcellular fractions. The diurnal variation of indole oxygenase in the leaves of *T. stans* is given in Figure 4. During the daytime, the activity of the enzyme was less, and it reached a peak during the nighttime. The maximum activity of the enzyme was found at 2 a.m.

DISCUSSION

The presence of a new enzyme system acting on indole in the leaves of *T. stans*, other than the previously reported indole oxidase, was discovered accidentally. Indole appearance from the reaction catalyzed by tryptophanase (6, 20) can be followed only in the absence of indole oxidase as the indole will be immediately converted to anthranil by the latter enzyme. Therefore, we wanted to inhibit the indole oxidase by known inhibitors. In our attempts to isolate tryptophanase from the leaves of *T. stans*, we had observed the rapid disappearance of indole in the presence of atebrin and diethyldithiocarbamate (unpublished results) which are strong inhibitors of indole oxidase (9). A perusal of the results reveals that indole oxygenase reported in this paper is entirely different from indole oxidase from the same source (9) and indole-oxidizing enzymes from other plant sources (2, 4).

Indole oxygenase from the leaves of *T. stans* was purified to homogeneity as evidenced by polyacrylamide gel electrophoresis and double immunodiffusion. The specific activity of the purified enzyme is 15.7 units/mg protein, while that of indole oxygenase from *J. grandiflorum* is 15 units/mg protein (4).

Indole oxygenase from *T. stans* has a mol wt of about 50,000 and is a single polypeptide chain, while indole oxygenase from *J. grandiflorum* (4) is a single polypeptide chain with a mol wt of about 40,000.

Indole oxygenase from *T. stans* shows an absolute requirement for molecular oxygen. Oxygen uptake studies showed utilization of 2 mol oxygen/mol indole oxidized. In the case of indole oxidase from the same source, it was reported that for every molecule of indole that disappears there was concomitant uptake of three atoms of oxygen: two atoms required for the cleavage of the indole ring and one for oxidation of *o*-aminobenzaldehyde to anthranil.

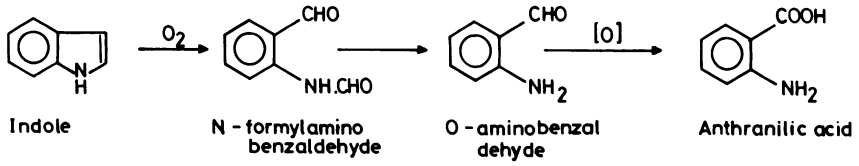

FIG. 5. Tentative scheme for the reaction catalyzed by indole ozygenase.

Anthranilic acid is the only detectable product of the reaction catalyzed by indole oxygenase from *T. stans*, and no anthranil could be detected. On the basis of product and oxygen requirement, the tentative scheme shown in Figure 5 was proposed for indole oxidation. Excellent stoichiometry was observed between indole disappearance and anthranilic acid formation. But in the case of oxygen consumption, one atom of oxygen could not be accounted for. Since four atoms of oxygen are consumed per molecule of indole oxidized and no other product could be detected, the fourth oxygen atom might be used in the formation of water. Formic acid, another proposed product, also could not be detected in the reaction mixture. Very good stoichiometry between indole disappearance, anthranilic acid formation, and oxygen consumption was observed in the case of indole oxygenase from *J. grandiflorum* (4). H_2O_2 is another product that was detected although no stoichiometry was observed between indole disappearance and H_2O_2 formation (4).

The enzyme activity could not be reconstituted by the addition of various cofactors and metal ions to the dialyzed enzyme. However, the enzyme activity was reconstituted by the addition of concentrated dialysate, suggesting that at least one of the cofactors is lost during dialysis. The enzymes from maize leaves (2) and *J. grandiflorum* (4), and indole oxidase from *T. stans* (9) also lose their activity on dialysis and reconstitution of the enzyme activity was achieved by the addition of FAD and Cu^{2+} in all three cases. These results suggest that indole oxygenase from *T. stans* is not a flavoprotein. Moreover, the visible and UV spectra of the protein show a single absorption peak at 408 nm apart from the typical protein absorption at 280 nm. Absence of a typical flavin absorption is consistent with indole oxygenase not being a flavoprotein. The negative atebrin data are also consistent with this interpretation.

Of the various metal ions tested, Fe^{2+} and Cu^{2+} inhibited the enzyme activity. Copper-specific chelators such as diethyldithiocarbamate, salicylaldoxime, bathocuproine, and neocuproine did not inhibit the enzyme activity, suggesting that the enzyme is not a cuproprotein, while all the similar indole-oxidizing enzymes from plant sources are drastically inhibited by Cu^{2+} chelators and are cuproproteins (2, 4, 9). Reconstitution experiments provide additional evidence for indole oxygenase not being a cuproprotein. Thus, indole oxygenase from *T. stans* is not a cuproflavoprotein in contrast to indole oxidases from *T. stans* and *Zea mays*, and indole oxygenase from *J. grandiflorum*.

Although the enzyme is inhibited by CO, the absence of typical Soret bands in the visible spectrum of CO-reduced enzyme (results not shown) rules out the possibility of heme as a cofactor in contrast to tryptophan-2,3-dioxygenase (17).

Indole oxygenase is not sensitive to either thiol compounds or thiol inhibitors, suggesting that no thiol group or disulfide bridge is essential for enzyme activity. On the other hand, indole oxygenase from *J. grandiflorum* is strongly inhibited by both sulfhydryl compounds and sulfhydryl reagents (4). Indole oxidase from *T. stans* was inhibited by thiol compounds and insensitive to thiol reagents (9).

This type of cleavage of the indole ring is similar to the reaction catalyzed by tryptophan peroxidase in which enzymically regenerated H_2O_2 is essential for the reaction (7). However, while the tryptophan peroxidase is inhibited by catalase, indole oxygenase is not susceptible to inhibition by catalase.

This enzyme is entirely different from indole oxidase reported from the same plant by Nair and Vaidyanathan. Indole oxidase from *T. stans* is a cuproflavoprotein and is atebrin sensitive (9) while indole oxygenase is not. The product of the reaction catalyzed by the former enzyme is anthranil while only anthranilic acid but no anthranil could be detected in the reaction catalyzed by indole oxygenase. Thus, two enzymes exist in *T. stans* acting on the same substrate leading to different metabolic pathways of indole.

Investigations are underway to characterize the cofactors and to elucidate the exact mechanism of indole oxidation.

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